VILNIUS UNIVERSITY

NATALIJA KRESTNIKOVA

INVESTIGATION OF DIFFERENTIATED MUSCLE-DERIVED STEM CELL DEATH AND SURVIVAL SIGNALLING MECHANISMS

Summary of doctoral dissertation
Physical sciences, biochemistry (04 P)

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VILNIAUS UNIVERSITETAS

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Introduction

Nowadays targeted therapy is becoming more common in cancer and other serious diseases treatment. Protein kinase inhibitors is a new class of specific drugs less toxic than traditional chemotherapic drugs. By now over 30 small-molecule drugs modulating kinase activity have undergone the first stage of clinical trials. Over 80 drugs are still passing pre-clinic tests.

Mitogen-activated protein kinases (MAPKs) are an obvious target for cancer and other diseases therapy. This highly conserved protein family is one of the major systems participating in transduction of extracellular signals to nucleus and other intracellular targets. The members of MAPK family are involved in regulation of a large variety of cellular processes such as cell growth, differentiation, development, death and survival (Ravingerova et al., 2002). Activation of MAPKs family plays a key role in tumorogenesis as well as in other pathological conditions.

During application of various cancer treatment strategies, e.g. conventional chemotherapy or target therapy, it is important to maintain adult stem cells residing in various organs and required for adult organism normal functioning. However, little is currently known about adult stem cell death mechanisms, cell response to combined chemo- and target therapy treatment. The evaluation of such cellular apoptosis/survival mechanisms could improve their survival during anti-cancer treatment.

Recently, the interest in use of stem cells for *in vitro* drug toxicity studies has dramatically grown. Stem cell-derived models are being used in various branches of toxicology. The study of drug-induced toxicity molecular machinery and elucidation of cellular-response pathways in toxicity studies are supposed to offer promising approaches in combining signal molecule-targeted and conventional therapies. However differences in cell death signalling regulation exist between immature and adult cells (Leppa et al, 2001; Zhang et al, 2007; Konorev et al, 2008). Therefore, for maintaining stem cell viability during appropriate drug therapy thorough knowledge of apoptosis-regulating signalling pathways determining sensitivity or resistance of adult stem cells is of great importance.

The aim of this work is to evaluate death and survival signalling mechanisms of differentiated adult muscle-derived stem cells.

The tasks of this work were as follows:

- to assess differentiation potential of adult rabbit muscle-derived cell lines;
- to compare sensitivity of differentiated and proliferating cells to apoptotic stimuli;
- to evaluate the role of Akt kinase in apoptosis regulation of differentiated cells;
- to determine MAPKs activation characteristics in differentiated cells after exposure to cytotoxic agent;
- to ascertain the role of MAPK in regulating death/survival of differentiated cells;
- to evaluate a possible role of MAP kinase JNK in regulating extrinsic (receptor) and intrinsic (mitochondrial) pathways and Akt kinase activation.

Novelty

Chemotherapeutic treatment may affect not only cancer cells, but also normal cells in the organism. Evaluation of adult tissue stem cell apoptosis/survival mechanism may improve cell survival during anti-cancer treatment.

In the present study we demonstrated that adult rabbit muscle-derived cell lines with an unlimited proliferative potential *in vitro* are able to differentiate into myogenic, adipogenic, osteogenic and neurogenic lineages.

This work was devoted to investigation of death and survival signalling mechanisms of differentiated adult-muscle derived stem cell culture. For the first time there was assessed sensitivity of differentiated adult muscle-derived stem cells to cisplatin, doxorubicin and also to oxidative stress inducers, namely, hydrogen peroxide and nitric oxide donor NOC-18. Cytotoxicity studies revealed that differentiated cells, except neurogenic, are more resistant to apoptosis inducers than proliferating cells. Our experiments defined the role of Akt and MAP kinases in regulating differentiated cell death/survival. The data obtained show an antiapoptotic action of PI3K/Akt signalling pathway as well as mitogen-activated kinase ERK and p38 in differentiated cells exposed to cytotoxic treatment. It was demonstrated that the level of Akt phosphorylation may be responsible for different susceptibility to death stimuli of Myo cells with distinct differentiation status. In this study we also found that the role of JNK MAP kinase in regulating muscle-derived stem cell apoptosis may change from proapoptotic to antiapoptotic during cell differentiation process. The results suggest that activation kinetics of stress kinase JNK and p38 does not determine the cell fate in response to apoptotic treatment.

The mechanisms of antiapoptotic action of JNK in differentiated cells were also clarified in this study. The results show that in differentiated cells JNK is involved in negative regulation of mitochondrial apoptosis pathway and positive regulation of Akt kinase activation.

Determination of new molecular targets involved in regulation of differentiating adult stem cell survival may lead to development of new therapeutic strategies increasing stem cell survival during chemotherapeutic treatment.

Defensive statements:

- Muscle-derived stem cells with unlimited proliferative potential *in vitro* are multipotent stem cells exhibiting myogenic, adipogenic, osteogenic and neurogenic differentiation;
- Muscle-derived stem cell susceptibility to apoptotic treatment changes during differentiation;
- Susceptibility of differentiated cells to apoptosis inducers may depend on Akt kinase phosphorylation levels;
- Activation kinetics of stress activated kinases p38 and JNK does not determine their role in regulation of apoptosis in muscle-derived stem cells exposed to apoptotic treatment;
- During cell differentiation JNK role in regulating muscle-derived stem cell apoptosis may change from proapoptotic to antiapoptotic;
- Antiapoptotic function of JNK in differentiated cells may be associated with negative regulation of mitochondrial apoptosis pathway and positive regulation of Akt activation.

Materials and Methods

Materials. Dulbecco's modified Eagle medium (DMEM), DMEM/F12, N2 supplement, Iscove's Modified Dulbeco medium (IMDM), horse serum, fetal bovine serum (FBS) were purchased from Invitrogen/Life Technologies (USA). Paraformaldehyde, PVDF membrane were purchased from Carl Roth GmbH&Co.KG (Germany). The cell culture plates were acquired from Orange Scientific (Belgium). The horseradish peroxidase-conjugated secondary antibodies (goat anti-rabbit and goat anti-mouse) were obtained from BioRad Laboratories, Inc. (USA), AlexaFluor 488 and 594-conjugated Goat Anti-Mouse IgG – from Molecular Probes. Anti-CD34-PE, Sca-1-PE, anti-desmin mouse monoclonal antibody were purchased from Abcam (UK), anti-M-cadherin, c-Jun, FasL – from BD Inc. (USA), anti-AKT pT308 – from Life Technologies Corp. (USA), beta-III-tubulin – from Chemicon (USA). Anti-myogenin mouse monoclonal antibody, Hank's balanced salt solution, trypan blue, NOC-18, cisplatin, oil Red O, Alizarin Red and most of other materials were from Sigma-Aldrich Inc. (USA). Phosphatidylinositol 3-kinase (PI3K) inhibitor Ly294002, other inhibitors U0126, SP600125, SB203580 were purchased from CalBiochem/MERCK (USA).

Cell culture. Myo cell lines Myo9, Myo11, and Myo28 were derived from individual adult rabbit thigh muscle anterior tibia, as described in Bukelskiene et al., 2005. The approval to use laboratory animals for the stem cell research (No. 0121, 2004-07-09 and 0171, 2007-10-31) was issued by Lithuanian Food and Veterinary Office. A piece of muscle tissue (0.03 cm^3) was placed on the plate with cold DMEM and minced with scissors into fragments less than 1 mm^3. The minced tissue was exposed to digestive solution containing 0.125% trypsin–EDTA, 1 mg/mL collagenase type V and 0.3 mg/mL hyaluronidase in phosphate buffered saline (PBS). The cell mass was incubated for 15 min at 37ºC in a shaker bath. After the enzyme treatment, cells were separated by filtration and centrifugation to remove components other than the cells, then washed with IMDM supplemented with 10% FBS and antibiotics: penicillin – 100 U/mL, streptomycin 100 μg/mL, referred as growth medium (GM). After, the cells were counted, their viability was evaluated by a trypan blue (0.4% trypan blue in PBS) exclusion test and plated to the tissue culture flasks. After 24-48 h, the non-adherent cells were transferred to a fresh flask. The replating procedure was repeated over a period of 7-8 days to isolate the slowly adhering cell population.

The obtained culture was maintained in GM, the cells were routinely passaged twice a week applying trypsin and EDTA mixture (*Biological Industries,* Izrael). Cells for the experiments were used from passages 20 to 60.

Clonal Myo line isolation. Myo cells were cloned using the method of limiting dilution, in which the Myo28 line cells were seeded at a low density in culture flasks and grown for 10 days until colonies appeared. Then, the cells from individual colonies were picked up, transferred to 10-cm^2 tissue culture plates and allowed to proliferate. A large expansion of cloned cells was achieved using regular cell growth protocol.

Induction and assessment of myogenic differentiation Cells were plated at $10^4/cm^2$ and cultured in GM allowing them to reach 70-80% confluence. To induce differentiation, cells were washed with PBS and cultured in DMEM containing antibiotics and 2% heat-inactivated horse serum for 4-6 days. Control Myo cells were grown in GM. Cell fusion index – nuclear number of multinucleated cells versus total nuclear number, was determined by phase contrast microscope (Nikon Eclipse TS 100). Western blot method was used to evaluate myogenin, a marker for the entry of myogenic cells into the differentiation pathway, expression during process of Myo cell myogenic differentiation. The differentiation potential of three different Myo cell lines was investigated, and representative data obtained with Myo9 cell line are presented.

Induction and assessment of adipogenic differentiation. Myo cell adipogenesis was induced by two methods. The first one – the use of adipogenic medium (DMEM, 10% FBS, 100 U/mL penicillin, 100 mg/mL streptomycin, 1 mM dexamethasone, 0.5 mM 3-isobutyl-1-

methylxanthine, 10 mg/mL insulin, 100 mM indomethacin) at 80% confluence stage of cell growth. Cells were grown for 7-14 days periodically changing adipogenic media and monitoring intracellular accumulation of lipids microscopically. Control Myo cells were simultaneously grown in regular GM. The differentiated Myo cell cultures were stained with Oil Red O dye for lipid content as described by Ramirez-Zacarias et al. (1992). Briefly, the cultures were rinsed in PBS to remove medium components and fixed in 4% paraformaldehyde at RT for 15 min. The cell samples were thoroughly rinsed with water and stained with Oil Red O (prepared in isopropanol) for 10 min. Intracellular red-stained fat droplets were analyzed by phase contrast microscopy. The second method used γ-linolenic acid Myo cells were cultured in DMEM supplemented with 10% FBS, 4.5 mg/mL glucose, and 100 mM gama-linolenic acid for 4 days (Wada et al., 2002; Hashimoto et al., 2006).

Induction and assessment of osteogenic differentiation. Myo cell osteogenesis was induced with supplements: 5×10^{-8} M dexamethasone, 160 mM L-ascorbic acid 2-phosphate, and 10 mM beta-glycerophosphate (Blum et al., 2004). The cells were cultured for 7 days changing osteogenic media, whereas control cells were grown in GM. After differentiation, the cells were washed with PBS and fixed in 4% paraformaldehyde at RT for 15 min. Finally, the cells were rinsed twice with pure water and stained with 2% Alizarin Red (pH 4.1–4.3). After staining for 5 min at RT, the monolayers were washed four times with pure water and examined by phase contrast microscope equipped with a CCD camera (QImaging ExiBlue).

Induction and assessment of neurogenic differentiation. Neural induction medium containing 1 mM retinoic acid, N2 in DMEM/F12, was used for neuron-like differentiation (Park et al., 2007). Differentiation was induced at 40–60% confluence. After 6 days in neural induction medium, the samples were examined for the presence of neuron-like cells by light microscope and differentiation was proved by the expression of beta- III-tubulin, as determined by immunocytochemistry and Western blot. To determine its expression, cells were washed twice with PBS and fixed in 4% paraformaldehyde for 15 min at RT. The samples were washed three times with PBS and permeabilised with 0.2% Triton X-100 in PBS for 15 min at RT; they were then washed three times with 1% BSA in PBS. Nonspecific binding was blocked by 1 h treatment in 10% goat serum, 1% BSA in PBS for 30 min.at 37°C. Cells were incubated with primary beta-III-tubulin antibody (dilution 1:2,000) for 1 h at 37°C, washed five times with 1% BSA in PBS, and incubated with Alexa Fluor 594-conjugated secondary antibody for 30 min. at 37°C. After rinsed five times with 1% BSA in PBS, samples were mounted in anti-fade medium and examined by a fluorescence microscope. Expression was also ascertained by Western blot assay as described below.

Flow cytometry. Untreated proliferating Myo cells were trypsinized, collected by centrifugation, suspended in PBS, washed twice and then fixed in ice-cold 70% ethanol. After centrifugation at 300× g for 5 min, the cells were suspended in PBS containing 2% FCS for 1h, then incubated with primary antibodies for at least 1 h at +4°C. Cells were washed twice with PBS containing 1% BSA and 1% goat serum, before the application of secondary AlexaFluor-488 antibodies in the same composition PBS. After incubation at +4°C for 30 min. in the dark, specimen was washed again and analyzed by flow cytometry using BD FacsCanto II.

Apoptosis assay. Cell apoptosis was determine using acridine orange (AO) and ethidium bromide (EB) fluorescent dyes. Cells were characterised as follows: V – viable (green chromatin fluorescence), VA – viable apoptotic with fragmented nuclei (green fragmented chromatin), NA – nonviable apoptotic with fragmented nuclei (orange fragmented chromatin). N – necrotic cells (orange nonfragmented nuclei). Chromatin-free cell lost their DNA and exhibited weak green-orange staining (Marcille and Massie, 1994).

Cell viability assay. Cell viability was measured using MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) assay. Water-insoluble MTT formazan was dissolved in DMSO and quantified spectrophotometrically reading absorbance at 570 nm in a Varioskan Flash plate reader (Thermo Scientific). In parallel trypan blue dye (0.4% trypan blue dye in PBS

(80 mM Na_2HPO_4, 100 mM NaCl)) exclusion test was used. Cell suspension was mixed with dye solution at 1:1 and cell was analyzed under the light microscope. Dye penetrates into nonviable cells and colours them blue.

Transfection. The expression construct for hemaglutinin-tagged version of constitutively active form of Akt in pBabe-puro vector (von Gise et al., 2001) was used for modulation of Akt protein kinase expression. Cells were plated at the density 5×10^4 cell/cm^2 in IMDM with 10% FBS without antibiotics. Transfection DNA and LIPOFECTAMIN^{TM}2000 reagent separately diluted in OPTI-MEM media (Invitrogen) according to manufacturer's recommendations. After 5 min of incubation, DNA was added to diluted LIPOFECTAMIN^{TM}2000. Mixed gently and incubated for 5 min at RT. Mixture was added to the cells and incubated for 6 h at 37°C. After incubation the media was replaced by IMDM containing 20% FBS. Three days later selective antibiotics were added.

Imunoblotting. Cells were lysed in ice-cold lysis buffer (10 mM TrisHCl pH 7.4, 50 mM NaCl, 5 mM EDTA, 50 mM NaF, 2 mM Na_3VO_4, with 1% Triton X-100 and supplemented with protease inhibitors aprotinin (20 μg/mL) and PMSF (1 mM). Protein concentrations were estimated by the Bradford assay, and equal amounts of protein were separated by SDS-PAGE on 10% acrylamide gels. Separated proteins were transferred onto PVDF membrane, and the blots were blocked by 5% low fat milk in TBST (5 mM Tris HCl pH 7.5, 0.1% Tween 20. 154 mM NaCl) for 1 h at room temperature (RT). The membranes were washed in TBST three times (5 min per wash), probed with the primary anti-myogenin antibody in 2% low fat milk in TBST for 24 h at 4 ºC, and then with the secondary antibody in the same 2% low fat milk in TBST for 1 h at RT. After the incubation, membranes were washed for 3 x 5 min in TBST. Proteins were then visualized by adding ECL reagents and exposing to X-ray film. To evaluate protein amounts, SDS-PAGE gels were stained with Invitrogen SimplyBlue Safe Stain. To confirm transfer onto a membrane, proteins were visualized with Pierce Reversible protein stain kit for PVDF membranes, using manufacturer‘s instructions.

Statistical analysis. Data are expressed as representative results or as a mean of at least three independent experiment ± SD. Statistical analysis was performed using paired Student t test. Differences were considered statistically significant at $p< 0.05$.

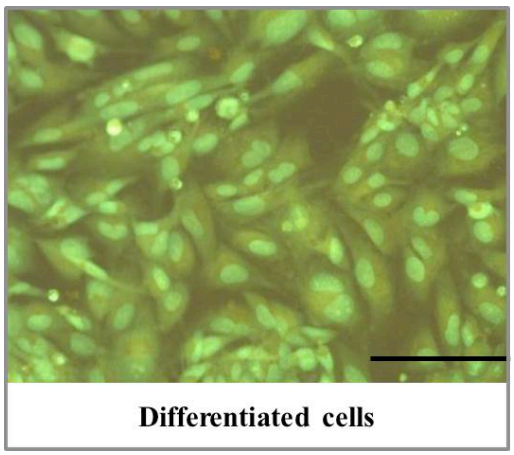
Results and Discussion

Characterisation of adult muscle-derived Myo cell lines. Characterisation of Myo cells maintained in culture for 20-60 passages using flow cytometry analysis identified the expression of stem cell surface antigen Sca-1 (13 ± 6%), hematopoietic progenitor cell antigen CD34 (65 ± 6%) as well as muscle progenitor cell marker, i.e. cell adhesion molecule M-cadherin (54 ± 7%), and intermediate filament protein desmin (87 ± 6%). The expression pattern slightly varied depending on the individual animal and cell state in culture. Muscle-derived progenitor cell markers expression is known to be able to vary reflecting the level of lineage progression in culture (Wu et al., 2010).

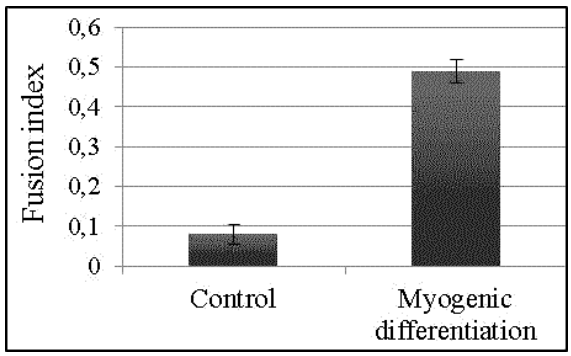
Myogenic differentiation of Myo line cells. As established earlier, adult rabbit muscle-derived cell lines (Myo) proved to have a long-time proliferation potential, i.e. cells could be maintained *in vitro* for over 10 months and have got a potential to differentiate into myogenic cells. Upon culturing in low serum medium Myo line cells differentiated into myocyte-like multinucleated cells expressing skeletal muscle specific myosin heavy chain protein, MHC (Bukelskiene et al., 2005; Baltriukienė, 2009).

We evaluated the cell fusion index and the expression of myogenin (muscle-specific transcription factor involved in the regulation of muscle cell development) (Fig.

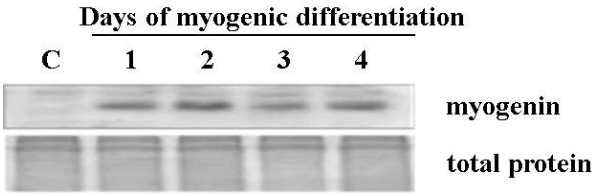
1 A-C). The obtained data prove that the amount of multinuclear cells and the level of myogenin expression increase as a result of induction of myogenic differentiation in Myo cells.



A



B



C

Fig.1. Myogenic differentiation of Myo cells. (A) Myo cells maintained in low serum condition fuse to form multinucleated muscle cells. Scale bar 75 μm. (B) Fusion index of control (proliferating cells) and differentiated cells population (random counts n=5, each more than 100 cells, $p<0.05$. (C) Western blot analysis of myogenin expression during myogenic differentiation: C – control, proliferating cells, 1-4 mark days of differentiation (see Materials and Methods section).

Muscle-derived Myo line cells maintained their myogenic potential during prolonged cultivation, which could make them useful in muscle regeneration, produce cells in the amounts for clinical application for the treatment of muscular disorders, e.g. muscular dystrophy or heart failure.

Adipogenic differentiation of Myo line cells. Muscle stem cells were previously considered to commitment to myogenic lineage, however in recent experiments these cells have demonstrated a broader capacity for differentiation not confined to myogenic cell lineage (Zammit et al., 2004; Asakura et al., 2001; Yamanouchi et al., 2007).

We investigated whether Myo line cells possess an adipogenic potential. One of the properties of adipogenic cells is the ability to accumulate lipids (Fernyhough et al., 2008). As shown in Figure 2A, the majority of cells exposed to special adipogenesis-inducing medium differentiated into the lipid droplets accumulating cells, whereas control Myo cells simultaneously maintained in regular growth medium did not show accumulation of lipid droplets.

Fatty acids are known to stimulate adipogenesis (Amri et al., 1991). We investigated the role of γ-linolenic acid on adipogenesis of Myo cells. The results demonstrated an increase of lipid bearing cells 4 days after addition of γ-linolenic acid to culture medium (Fig. 2 B).

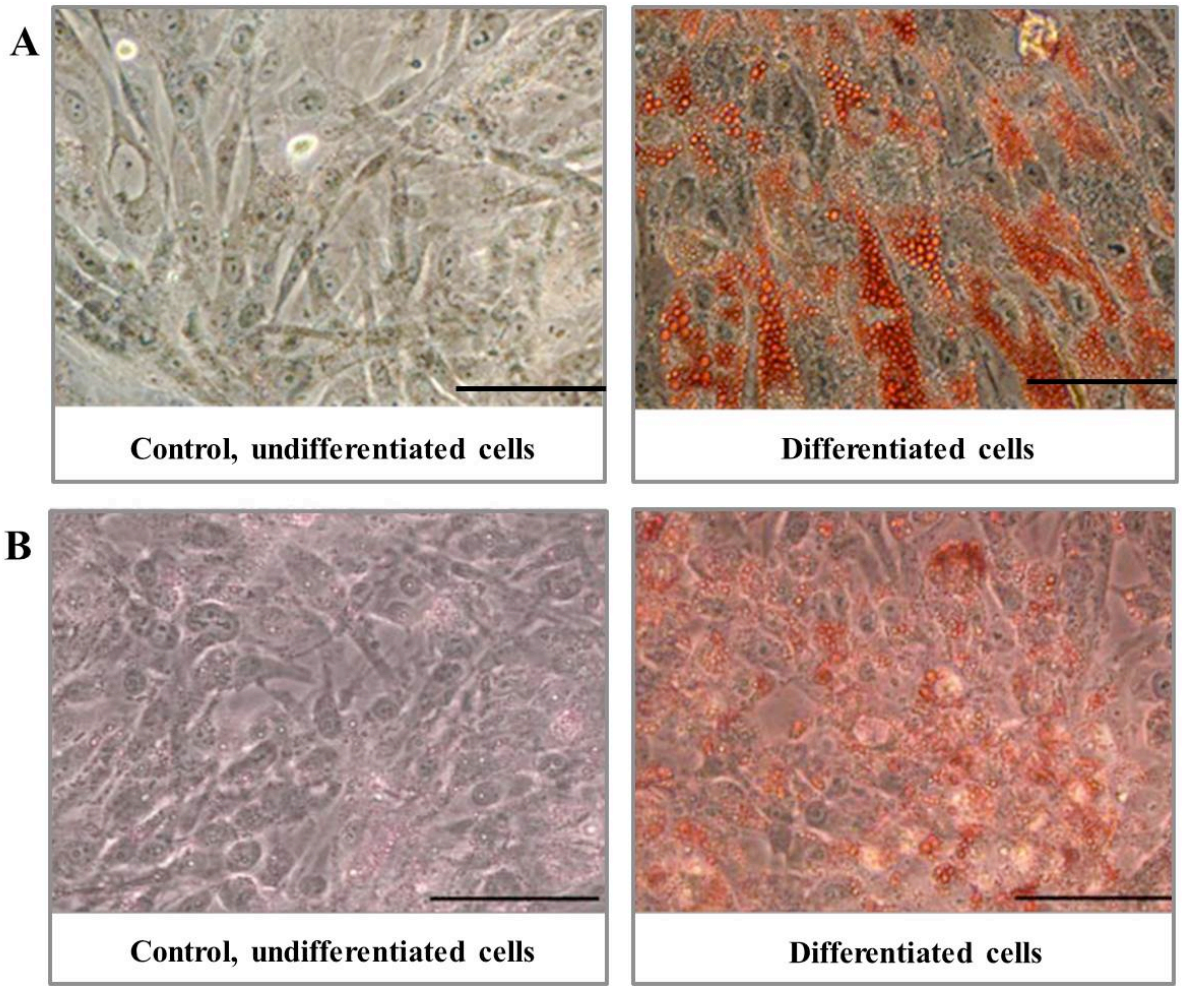


Fig. 2. Adipogenic differentiation of Myo cells. Oil Red O staining of lipid droplets in Myo cells after adipogenic differentiation induced by adipogenic media for 7 days (A) and γ-linolenic acid for 4 days (B) (See Materials and Methods). Control undifferentiated cells were grown in regular medium for 7 or 4 days, respectively. Photomicrographs represent at least, three independent experiments performed with three different Myo cell lines. Scale bars 50 μm.

The ability of MDSC to possess adipogenic potential could explain the presence of adipose tissue within skeletal muscle that occurs under various pathological conditions, for example metabolic diseases and age-related sarcopenia (Vettor et al., 2009).

Osteogenic differentiation of Myo line cells. To determine whether rabbit muscle-derived Myo cells are able to undergo osteogenic differentiation, we examined calcium deposition using osteogenic differentiation medium containing β-glicerophosphate as a source of phosphate ions (Maniatopoulos et al., 1988). Calcium deposition was detected with the calcium-staining dye Alizarin Red S. Development of mineralization nodules was determined in the Myo cell cultures grown in the osteogenic differentiation medium for 7 days. Alizarin Red S-positive aggregates of calcium mineralization were not detected in the control Myo cells not exposed to osteogenic induction (Fig. 3).

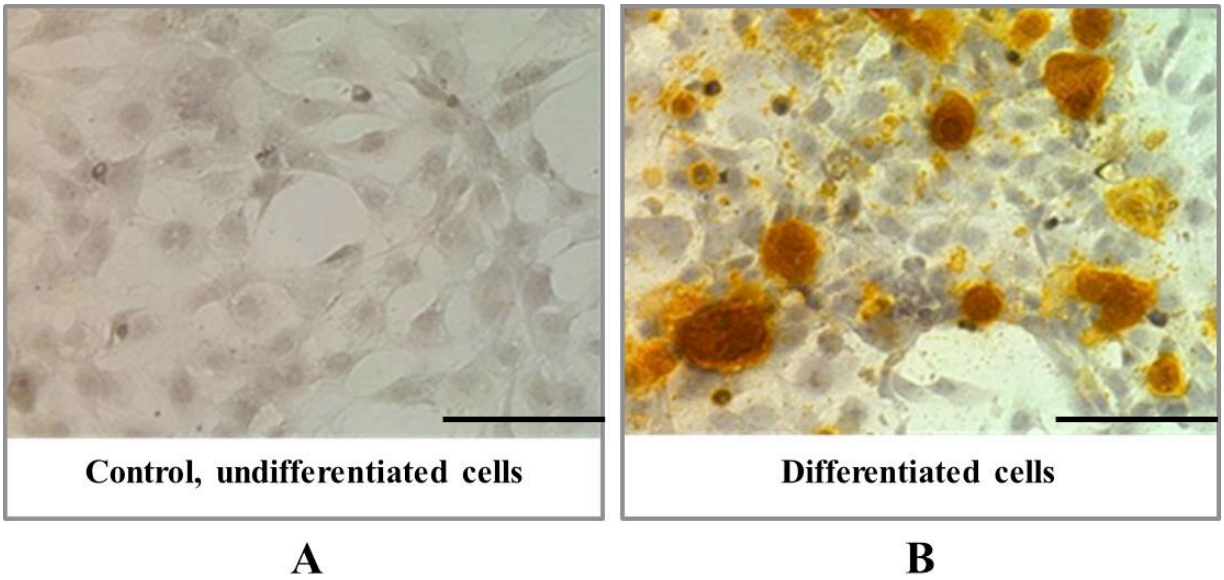


Fig. 3. Osteogenic differentiation of Myo cells. Alizarin Red stained control (A) and osteogenic cocktail-treated differentiated cells (B). Cells were exposed to differentiation media for 7 days (see Materials and Methods). Alizarin Red-positive aggregates of calcium mineralisation were not detected in control (not subjected to osteogenic induction) cells. Photomicrographs represent at least, three independent experiments performed with three different Myo cell lines. Scale bars 50 μm.

Neurogenic differentiation of Myo cells. Trans-differentiation of adult MDSC into neural-lineage cells can occur. Neuronal differentiation of stem cells isolated from adult muscle in vitro have been shown in human, mouse and rat models (Alessandri et al., 2004; Schultz et al., 2006; Zhang et al., 2006; Tamaki et al., 2007). Myo line cells after 6 days of exposure to neural induction medium containing retinoic acid, developed neuron-like morphology (Figure 4A–B), some of them expressing beta-III-tubulin protein (Figure 4B), being a microtubule component, expressed exclusively in neurons. Western blot analysis of Myo cell extracts also confirmed induction of beta-III-tubulin expression.

Multilineage differentiation of single-cell derived clone. Multipotency of cells from Myo lines was confirmed by cell cloning. The data presented in Figure 5A-D show that single cell-derived Myo28 line clone had the potential to differentiate into myocytes, adipocytes, osteocytes and neuron-like cells. Therefore, clonal analysis of Myo cells demonstrates that given an adequate stimulus a single cell from rabbit skeletal muscle can develop in many different directions and, consequently, it possesses multipotency (Kalvelyte et al., 2013).

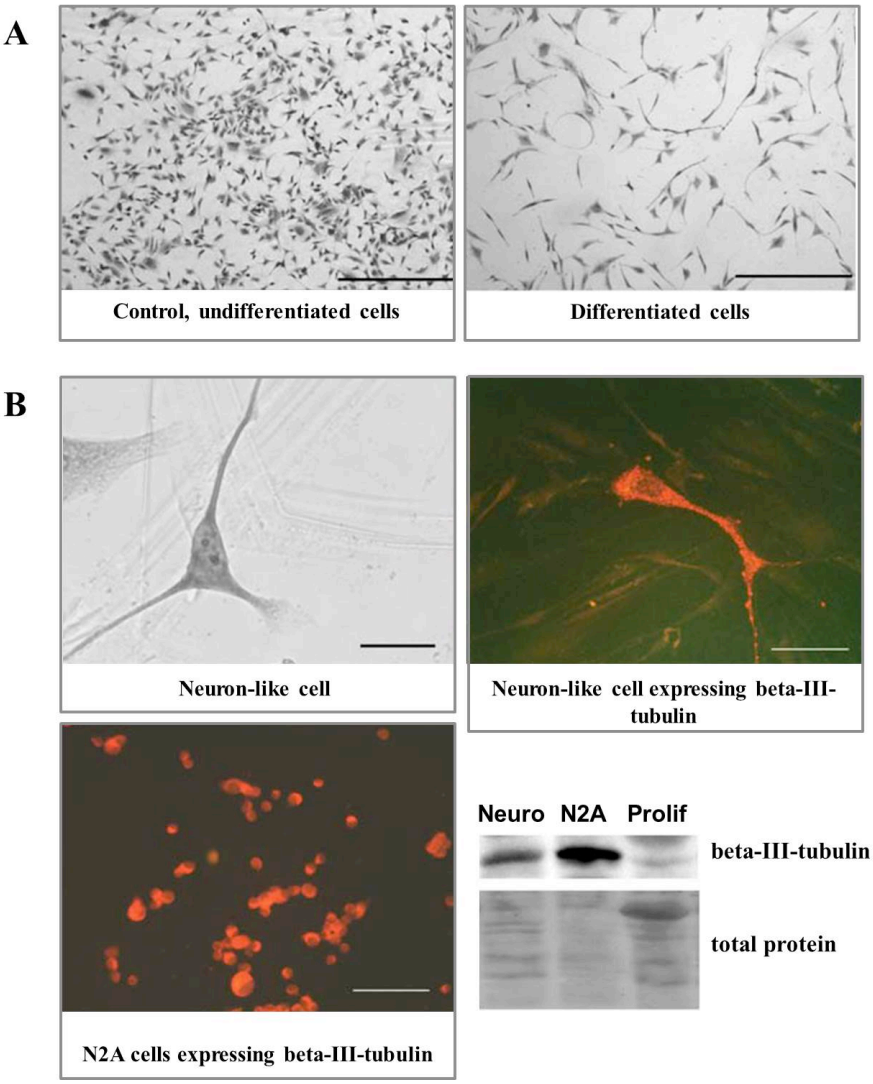


Fig. 4. Neurogenic differentiation of Myo cells. Photomicrographs of Myo cells: (A) control undifferentiated and differentiated cells, subjected for 6 days to neurogenic differentiation. The data represent at least three independent experiments performed with three different Myo cell lines. Scale bars 400 mm. (B) Myo cells exposed to neurogenic media possess neuron-like morphology and express beta-III-tubulin (red fluorescent labelling and Western blot). Neuroblastoma N2A cells expressing beta-III-tubulin were used as positive control. Scale bars 50 μm.

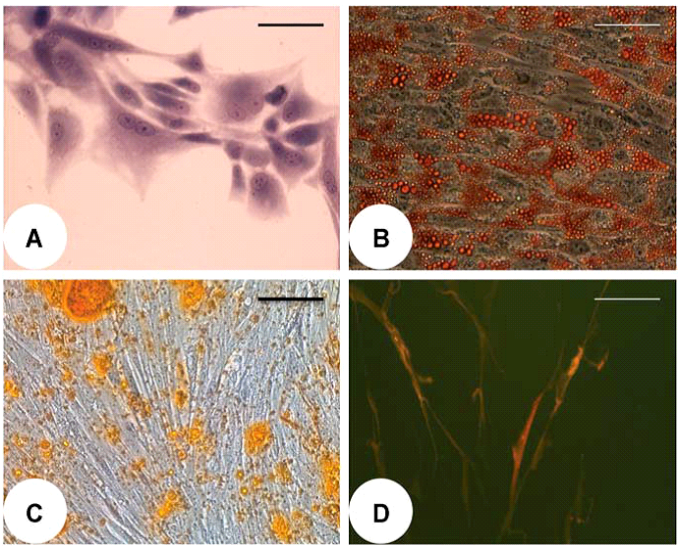


Fig. 5. **Multipotency of single cell-derived Myo cell clone.** (A) Multinuclear cells from Myo28 line clone during myogenic differentiation (duration of differentiation – 4 days). (B) Oil Red O stained cells during adipogenic differentiation after incubation with γ -linolenic acid for 3 days. (C) Alizarin Redstained cells during osteogenic differentiation (duration of differentiation – 7 days). (D) Expression of beta-III-tubulin in Myo28 cells exposed to neurogenic media for 6 days. Cells were differentiated as described in Materials and Methods Section. Photomicrographs represent at least three independent experiments showing similar results. Scale bars 50 μm.

Myo-. adipo- and osteogenically differentiated muscle-derived Myo cells are more resistant to cytotoxic treatment than proliferating cells. We have assessed the susceptibility of differentiated cells to various apoptosis inducers. For that purpose there was used chemotherapeutic agents (cisplatin, doxorubicin) and oxidative stress inducing agents (hydrogen peroxide and nitric oxide donor NOC-18). Our previous studies revealed that the doses of the above mentioned drugs reducing the initial proliferating cells number by 40-60% usually induced an apoptotic mode of cell death, which was confirmed by different methods, i.e. microscopically by the detection of cell morphological changes, cleavage of caspase-3 as well as DNA fragmentation and cell cycle analysis (Baltriukiene, 2009). Viability of differentiated and proliferating cells after 24 h of cytotoxic treatment was evaluated by MTT method. The results show that cells become more resistant to apoptotic treatment during their myogenic, adipogenic and osteogenic differentiation. However they become more susceptible to apoptosis during neurogenic differentiation (Fig. 6A-B).

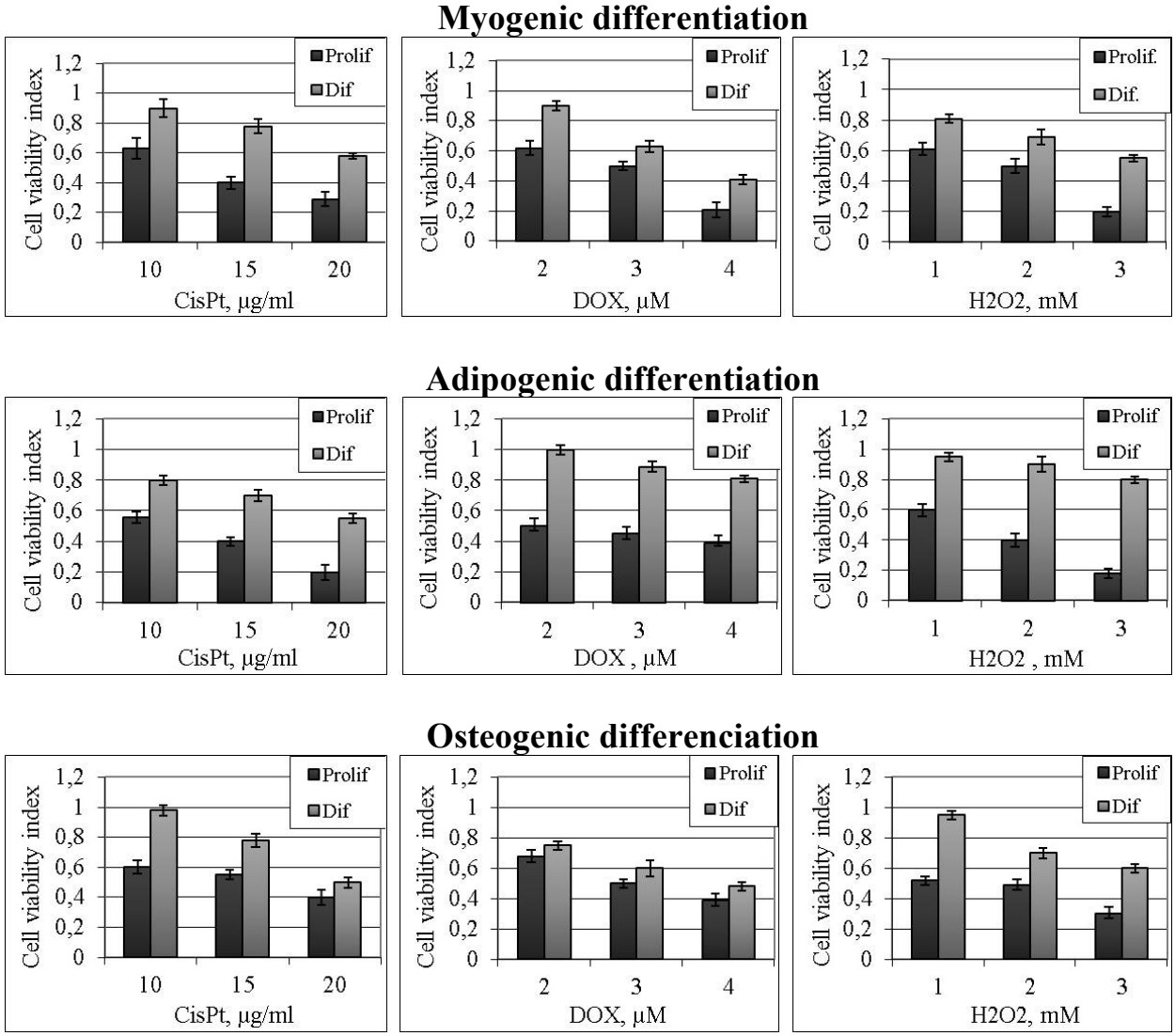


Fig. 6A. Myo-, adipo- and osteogenically differentiated Myo cells are more resistant to apoptotic treatment than proliferating cells. Prolif – control cells, 24 h in GM; Dif – various direction differentiated Myo cells. Cells were differentiated as described in Materials and Methods Section. Cell viability was measured by MTT assay. Results are expressed as ratio of viable cells after 24 h exposure to viable cells before treatment. Data are presented as the mean±SD of at least, three independent experiments, performed in triplicate; $p<0.05$.

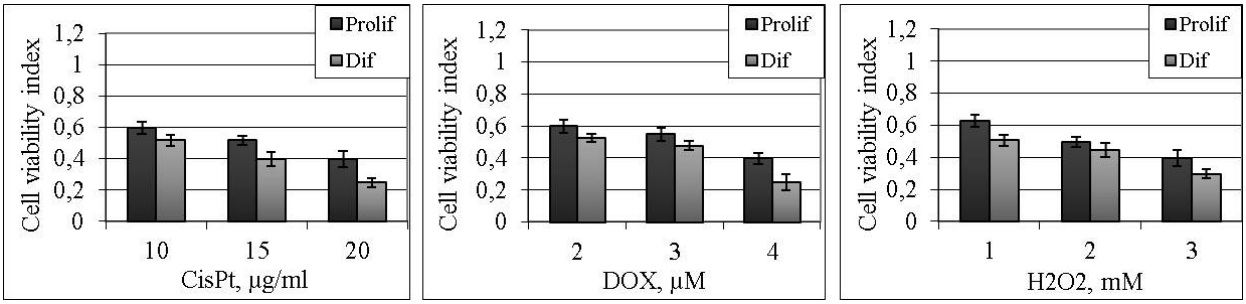


Fig. 6B. Neurogenically differentiated Myo cells are more susceptible to apoptotic treatment. Prolif – control cells, 24 h in GM; Dif – neurogenically differentiated Myo cells. Cells were differentiated as described in Materials and Methods Section. Cell viability was measured by MTT assay. Results are expressed as ratio of viable cells after 24 h exposure to viable cells before treatment. Data are presented as the mean±SD of at least, three independent experiments, performed in triplicate; $p<0.05$.

Akt kinase involved in survival of differentiated Myo cells. Akt is a serine/threonine kinase with diverse roles relating to the regulation of cell growth, proliferation, migration, glucose metabolism, transcription, protein synthesis, angiogenesis and cell survival. Akt has been demonstrated to inhibit apoptosis by many mechanisms, effecting diverse apoptotic pathways at multiple levels, from upstream signalling pathways which regulate transcriptional activity, to downstream targets, such as caspase-9 (Fujio et al., 1999; Brunet et al., 1999; van der Heide et al., 2006; Burke et al., 2007).

We investigated the potential role of Akt in Myo cell resistance to apoptosis. Our data presented in Figure 7 show increased levels of Akt phosphorylation in myo-, osteo- and adipogenically differentiated cells in comparison with proliferating cells. Conversely, Akt phosphorylation was downregulated in case of neurogenic differentiation.

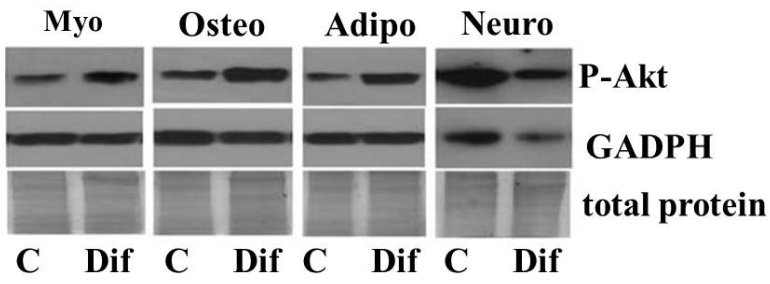


Fig. 7. Changes of phosphorylated Akt (pThr308) levels in differentiated cells. C – control, proliferating cells, Dif – differentiated cells, cultured in lineage-specific differentiation media. Akt phosphorylation was examined by Western blot analysis (see Materials and Methods Section).

To prove the involvement of protein kinase AKT in the resistance of differentiated Myo cells to apoptotic stimuli we used cisplatin as cell death-inducing agent, and .Akt upstream kinase PI3 inhibitor Ly294002. The efficiency of Ly294002 was verified by decreased phosphorylation of Akt at Thr308 in differentiated cells (Fig. 8A). We found that pretreatment of differentiated Myo cells with Akt inhibitor enhanced cell death (Fig. 8B). Therefore, our results show a protective role of Akt in cisplatin induced apoptosis of differentiated Myo cells. Thus, the levels of Akt phosphorylation may be responsible for different susceptibility to death stimuli of Myo cells with distinct differentiation status.

The pro-survival function of Akt in differentiated cell was also confirmed by genetic methods. Differentiated Myo cells transfected with puro-Babe-Akt and overexpressing Akt kinase (Stulpinas et al., 2012) were more resistant to cytotoxic cisplatin and doxorubicin treatment than control cells transfected with control puro-Babe-vect plasmids (Fig. 8C)

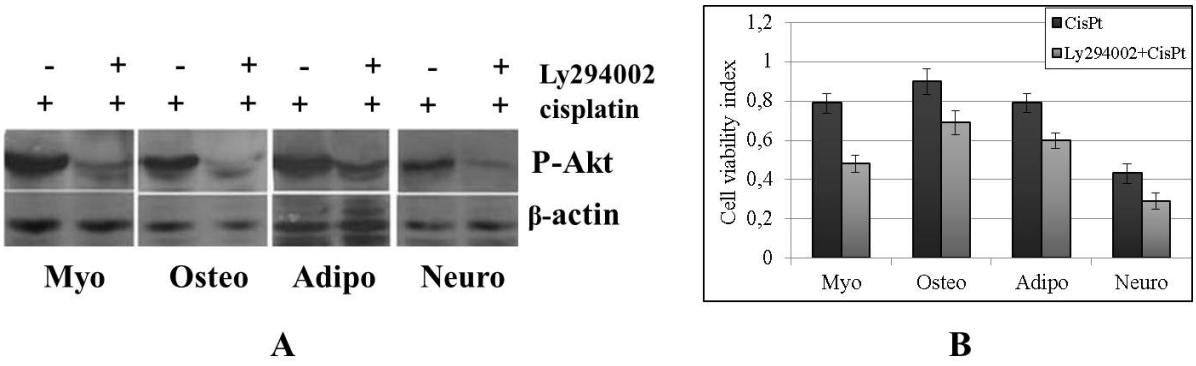


Fig. 8. The role of Akt in apoptosis regulation of differentiated Myo cells exposed to genotoxic treatment. (A) PI3 kinase inhibitor, Ly294002, decreases the level of pAkt in differentiated Myo cells. Akt phosphorylation (Thr308) was examined by Western blot analysis after 8 h of exposure (see Materials and Methods Section). (B) Ly294002 sensitises differentiated Myo cells to cisplatin-induced cell death. Cells were exposed to 10 μM Ly294002 for 15 min prior adding cisplatin (15 μg/ml). (C) Myogenically differentiated Myo cells overexpressing Akt are more resistant to cisplatin (CisPt) and doxorubicin (DOX) apoptotic treatment. Cell viability was assayed by MTT assay after 24 h treatment. Results are expressed as a ratio of the number of viable cells after indicated treatment to the number of cells in initial control (before treatment). The data are presented as mean±SD from at least three experiments performed in duplicate; $p < 0.05$.

MAP kinase pathway is involved in Myo cells response to cytotoxic stress. The initiation of programmed cell death by various anticancer drugs activates multiple signal transduction pathways, among which are the MAPK pathways (Brozovic and Osmak, 2007). These pathways are parallel cascades of structurally related serine/threonine kinases that play pivotal roles in transducing various extracellular signals to the nucleus. The MAPK signalling cascades regulate a variety of cellular activities, including cell growth, differentiation, survival, and death (Junttila et al., 2008). In mammals, MAPKs are divided into three major groups: ERKs, JNKs/stress-activated protein kinases, and p38, based on their degree of homology, biological activities, and phosphorylation motifs. Even though these signalling systems are built from evolutionarily related protein kinases, they produce distinct biological responses. The biological effects of MAPK signalling are executed by phosphorylation of downstream substrates, most notably a number of signal-responsive transcription factors. The broad range of these substrates indicates that MAPKs have pivotal roles in cellular signal transduction and suggests that

the extent and duration of MAPK activation play key roles in controlling cell functions (Hommes et al., 2003; Plotnikov et al., 2011; Mansouri et al., 2003).

This study was aimed at clarifying the role of MAP kinase in regulating apoptosis of differentiated muscle-derived stem cells. Cell contacts are known to be crucial for myogenic differentiation (Wu et al., 2000). Consequently, simultaneously with differentiated cells in the myogenic differentiation medium we cultivated confluent culture cells in the growth media for the same period of time. Both populations of cells were later exposed to apoptosis inducers. We investigated the effect of cisplatin on the phosphorylation of MAPKs in differentiated and confluent culture cells. The phosphorylation of ERK, p38 and JNK was assessed by using specific antibodies that recognize the phosphorylated (activated) forms of these proteins. The total amount of MAPKs was assessed using antibodies that recognize these proteins regardless of their phosphorylation status.

ERK kinase is involved in survival of muscle-derived cells. ERK kinase is known to be an important regulator of cell growth and survival. Some studies implicate ERK1/2 in regulation of myogenesis (Jones et al., 2001). The results show that cisplatin induced late and sustained phosphorylation of ERK in myogenically differentiated and confluent culture cells (Fig. 9A). The role of ERK in cell apoptosis induced by cisplatin was studied using U0126, inhibitor of MEK1/2 acting upstream ERK. The obtained data prove the protective role of ERK in differentiated and confluent Myo cell apoptosis induced by cisplatin (Fig. 9B).

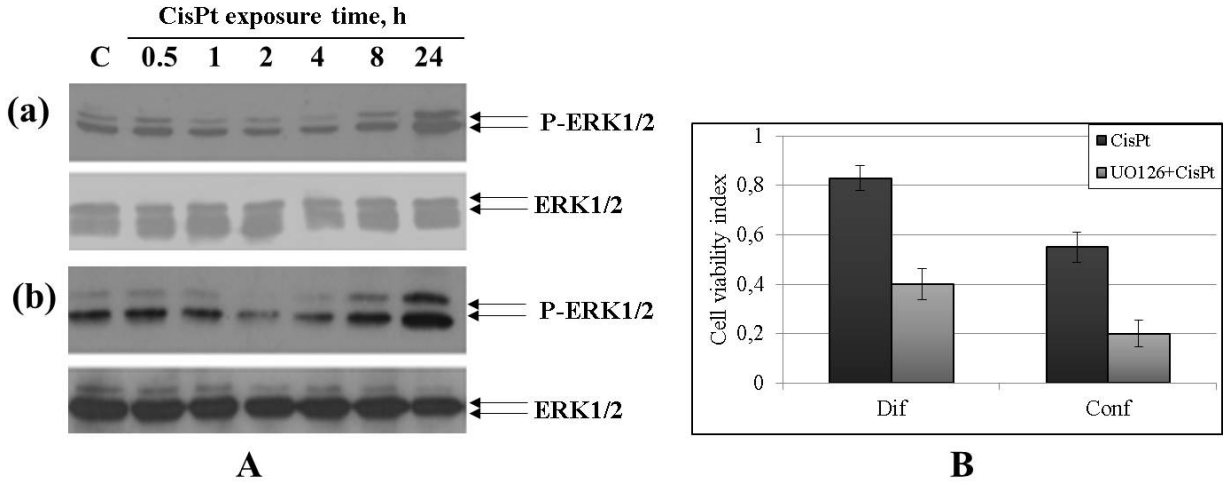


Fig. 9. The role of ERK kinase in differentiated and confluent cell apoptosis induced by cisplatin. (A) Time course of phosphorylation and expression of ERK during cisplatin treatment. Differentiated (a) and confluent (b) cells were exposed to cisplatin (15 μg/ml) and analysed at indicated time points. ERK phosphorylation was analysed by Western blotting with anti-phospho-ERK antibodies. The total amount of ERK was assessed using antibodies that recognize these proteins independent of their phosphorylation. (B) The effect of MEK1/2 inhibitor U0126 on cell viability. Cells were exposed to 20 μM of U0126 15 min prior to cisplatin (15 μg/ml). Cell viability was measured by MTT assay after 24 h of exposure. Results are expressed as a ratio of the number of viable cells after indicated treatment to the number of initial control cells (before treatment). The data are presented as mean±SD from at least three experiments performed in duplicate; $p < 0.05$.

p38 kinase is involved in survival of differentiated muscle-derived stem cells. The role of p38 in regulating cell apoptosis is rather controversial. It was reported that p38 activation can enhance cardiac myocyte survival (Craig et al.,2000). However a number

of studies propose that that p38 activation actually induces cardiac myocyte apoptosis (Kang et al., 2000; Mackay et al., 2000). The results of this study show an increased and sustained phosphorylation of p38 in differentiated and confluent culture cells exposed to cisplatin. No changes in expression of p38 were found (Fig. 10A). Sustained p38 activation is normally associated with induction of apoptosis (Wang et al., 2004). In order to evaluate the role of p38 in cisplatin induced apoptosis of differentiated and confluent Myo cells we used specific p38 inhibitor SB203580. The results prove that p38 inhibition increases cell sensitivity to apoptotic treatment and that during Myo cell differentiation and confluent growing p38 kinase acts as an antiapoptotic molecule. (Fig. 10B).

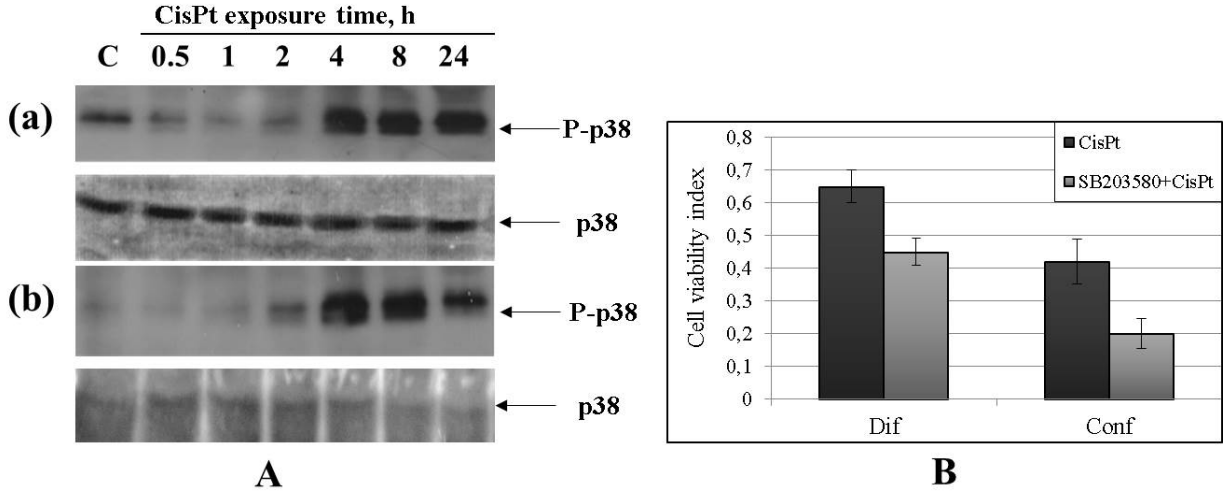


Fig. 10. The role of p38 kinase in differentiated and confluent cells apoptosis, induced by cisplatin. (A) Time course of phosphorylation and expression of p38 during cisplatin treatment. Differentiated (a) and confluent (b) cells were exposed to cisplatin (15 μg/ml) and analyzed at indicated time points. P38 phosphorylation was analysed by Western blotting with anti-phospho-p38 antibodies. The total amount of p38 was assessed using antibodies that recognize these proteins independent of their phosphorylation. (B) The effect of p38 inhibitor SB203580 on cell viability. Cells were exposed to 20 μM of SB203580 15 min prior to cisplatin (15 μg/ml). Cell viability was measured by MTT assay after 24 h of exposure. Results are expressed as a ratio of the number of viable cells after indicated treatment to the number of cells in initial control (before treatment). The data are presented as mean±SD from at least three experiments performed in duplicate; $p< 0.05$.

Opposite role of JNK kinase in regulation of apoptosis in differentiated and proliferating Myo cells. JNK signal transduction pathway is usually activated by various stress factors including genotoxins (Benhar et al., 2002; Helbig et al., 2011). However the role of JNK in stress is unclear. JNK kinase pathway has been reported to implicate in both apoptosis and survival signalling. Specific role of JNK may therefore depend on the cellular context (Davis et al., 2000).

The obtained results demonstrate a gradual and prolonged increase of JNK and its target protein transcription factor c-Jun phosphorylation in differentiated and confluent cells exposed to cisplatin. Phosphorylation of JNK and c-Jun was significantly elevated within 4-24 hours after the cell exposure to cytotoxic agent (Fig. 11A-B)

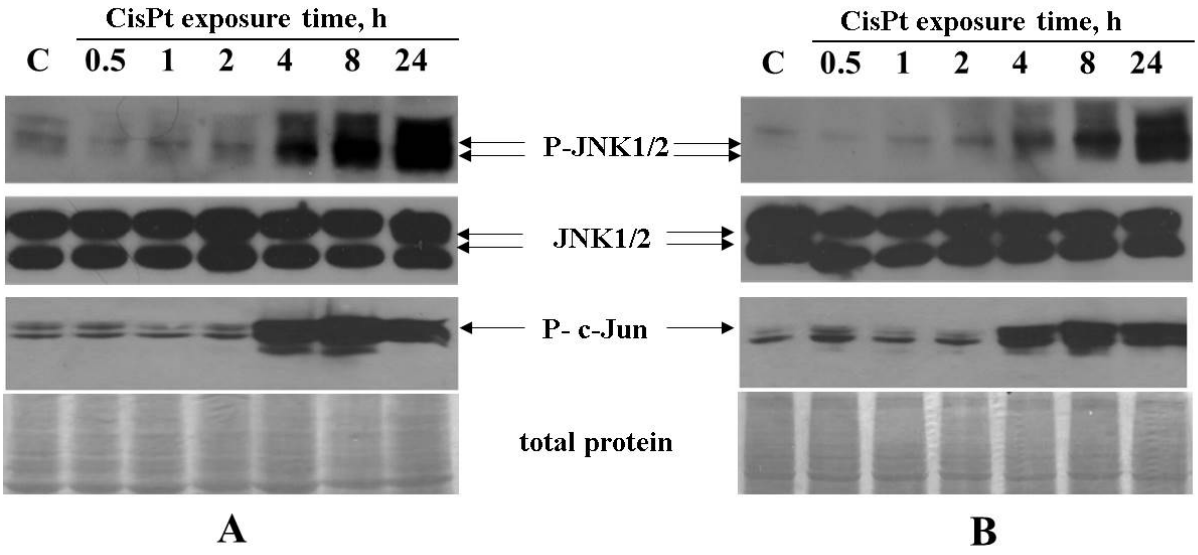


Fig. 11. Time course of JNK and c-Jun phosphorylation in response to cisplatin. Differentiated (A) and confluent (B) cells were exposed to cisplatin (15 μg/ml) and analysed at indicated time points. JNK and c-Jun phosphorylation was analysed by Western blotting with anti-phospho-JNK and anti-phospho-c-Jun antibodies. The total amount of JNK was assessed using antibodies that recognize these proteins independently of their phosphorylation.

Further we investigated the role of activated JNK in differentiated and confluent Myo cell apoptosis applying JNK inhibitor SP600125, the efficiency of which was confirmed by decreased phosphorylation of c-Jun (Fig. 12).

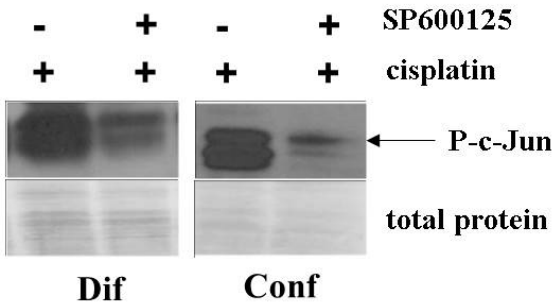


Fig. 12. Effect of JNK inhibitor SP600125 on c-Jun phosphorylation. Cells were pretreated with 40 μM SP600125 30 min. prior to 15 μg/ml cisplatin. Phosphorylation of c-Jun was analysed by Western blotting with anti-phospho-c-Jun (Ser63) antibodies after 8 h of exposure.

Notably, in this study opposite anti-apoptotic JNK function was found in differentiated and confluent culture cells versus pro-apoptotic in proliferating cells confirmed in previous studies (Baltriukienė, 2009). Pretreatment with JNK inhibitor was found to significantly enhance differentiated and confluent death of cell exposed to cisplatin (Fig. 13 A-B).

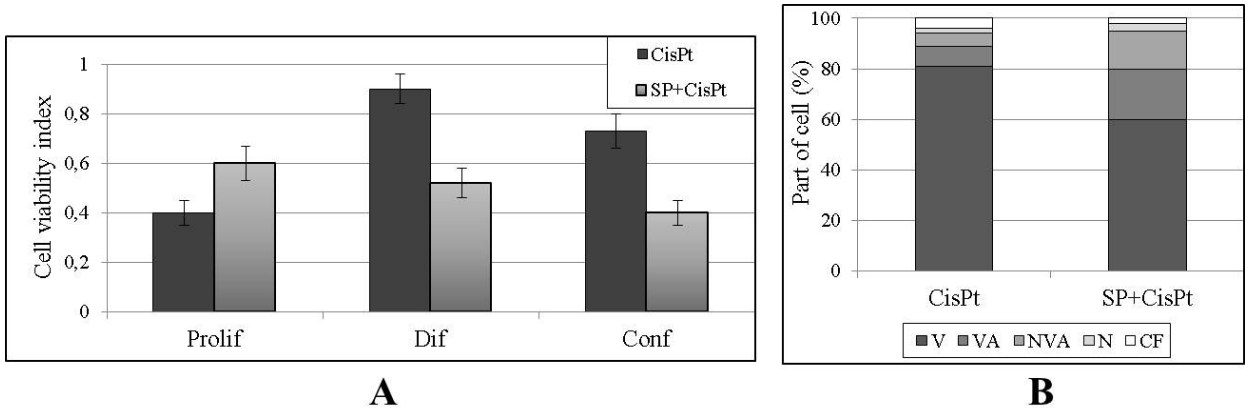


Fig. 13. The effect of JNK inhibitor SP600125 on cisplatin induced Myo cell death. (A) Effect of SP600125 on cell viability. Proliferated (Prolif), differentiated (Dif) and confluent culture (Conf) cells were exposed to 40 μM of SP600125 30 min. prior to cisplatin (15 μg/ml). Cell viability was measured by MTT assay after 24 h of exposure. Results are expressed as a ratio of the number of viable cells after appropriate treatment to the number of cells in initial control (before treatment). The data are presented as mean±SD from at least three experiments performed in duplicate; $p < 0.05$. (B) Distribution of V – viable, VA – viable apoptotic, NVA – nonviable apoptotic, N – necrotic, CF – chromatin free cells in differentiated Myo cells population after 24 h of 15 μg/ml cisplatin exposure.

Such results were also shown on other Myo cell lines (Myo11, Myo26 and Myo28), proving the protective role of JNK in cisplatin induced myogenically differentiated and confluent cell apoptosis (Fig. 14).

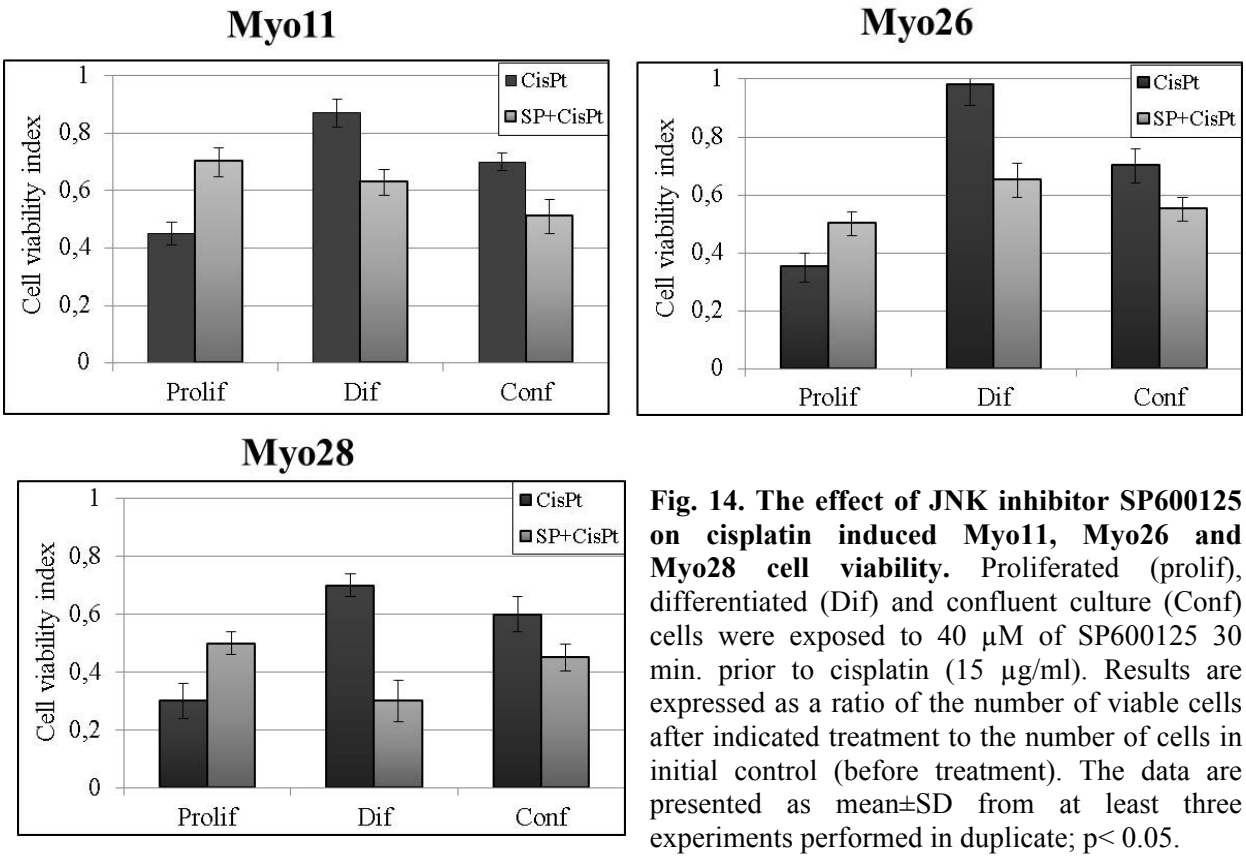


Fig. 14. The effect of JNK inhibitor SP600125 on cisplatin induced Myo11, Myo26 and Myo28 cell viability. Proliferated (prolif), differentiated (Dif) and confluent culture (Conf) cells were exposed to 40 μM of SP600125 30 min. prior to cisplatin (15 μg/ml). Results are expressed as a ratio of the number of viable cells after indicated treatment to the number of cells in initial control (before treatment). The data are presented as mean±SD from at least three experiments performed in duplicate; $p < 0.05$.

We also investigated whether JNK antiapoptotic action in differentiated and confluent cells would be the same with other apoptosis inducers. For that purpose we used chemotherapeutic/genotoxic agent doxorubicin and oxidative stress inducer nitric oxide donor NOC-18.

Similarly gradual and prolonged activation of JNK in differentiated cells was found after doxorubicin treatment. JNK role change from proapoptotic in proliferating to antiapoptotic in differentiated Myo cells was also proved (Fig. 15).

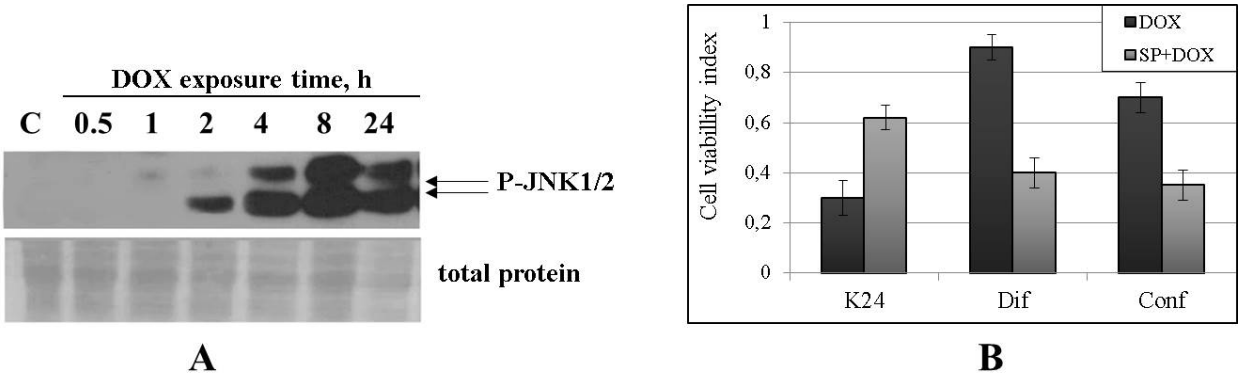


Fig. 15. The role of JNK in Myo cell apoptosis, induced by doxorubicin. (A) Time course of phosphorylation of JNK during doxorubicin (DOX) treatment. Differentiated cells were exposed to doxorubicin (2.5 μM) and analyzed at indicated time points by Western blotting. (B) Effect of JNK inhibitor SP600125 on cell viability. Cells were exposed to 40 μM of SP600125 30 min. prior to doxorubicin (2.5 μM). Cell viability was measured by MTT assay after 24 h of exposure. Results are expressed as a ratio of the number of viable cells after indicated treatment to the number of cells in initial control (before treatment). The data are presented as mean±SD from at least three experiments performed in duplicate; $p< 0.05$.

The same JNK phosphorylation pattern was found in NOC-18 treated differentiated cells (Fig. 16A). Our studies (Bironaitė at al., 2009) also showed prolonged NOC-18 induced JNK activation in proliferating Myo cells, which was associated with cell survival. Pretreatment of differentiated cells with SP600125 resulted in enhanced cell death (Fig. 16B) The results of apoptotic studies proves increased apoptosis morphological changes in differentiated (a) and proliferating (b) cell populations induced by pretreatment with SP600125 (Fig. 16C).

The results show the protective role of JNK in proliferating and differentiated cells during NOC-18 induced apoptosis. As known from various sources, the role of activated JNK during oxidative stress is rather controversial. It was shown that active JNK acts as a proapoptotic molecule during oxidative stress induced cardiac myocyte apoptosis. As known from literature, JNK induces the release of pro-apoptotic molecules such as cytochrome c and AIF from mitochondria (Aoki et al., 2002). However other data indicate that JNK activation, rather than promoting oxidative stress induced apoptosis, in fact, is cytoprotective (Andreka et al., 2001; Dougherty et al., 2002; Minamino et al., 1999).

It was suggested that the time course of JNK activation is critically important for signal transduction; for example, apoptotic signalling by JNK requires sustained activation while transient activation can signal cell survival (Ventura et al., 2006; Mansouri et al., 2003; Marchall, 1995). However our results contradict this notion. Thus

our data indicate that depending on cell differentiation stage and apoptotic inducer the activated JNK may cause apoptosis and cell survival as well.

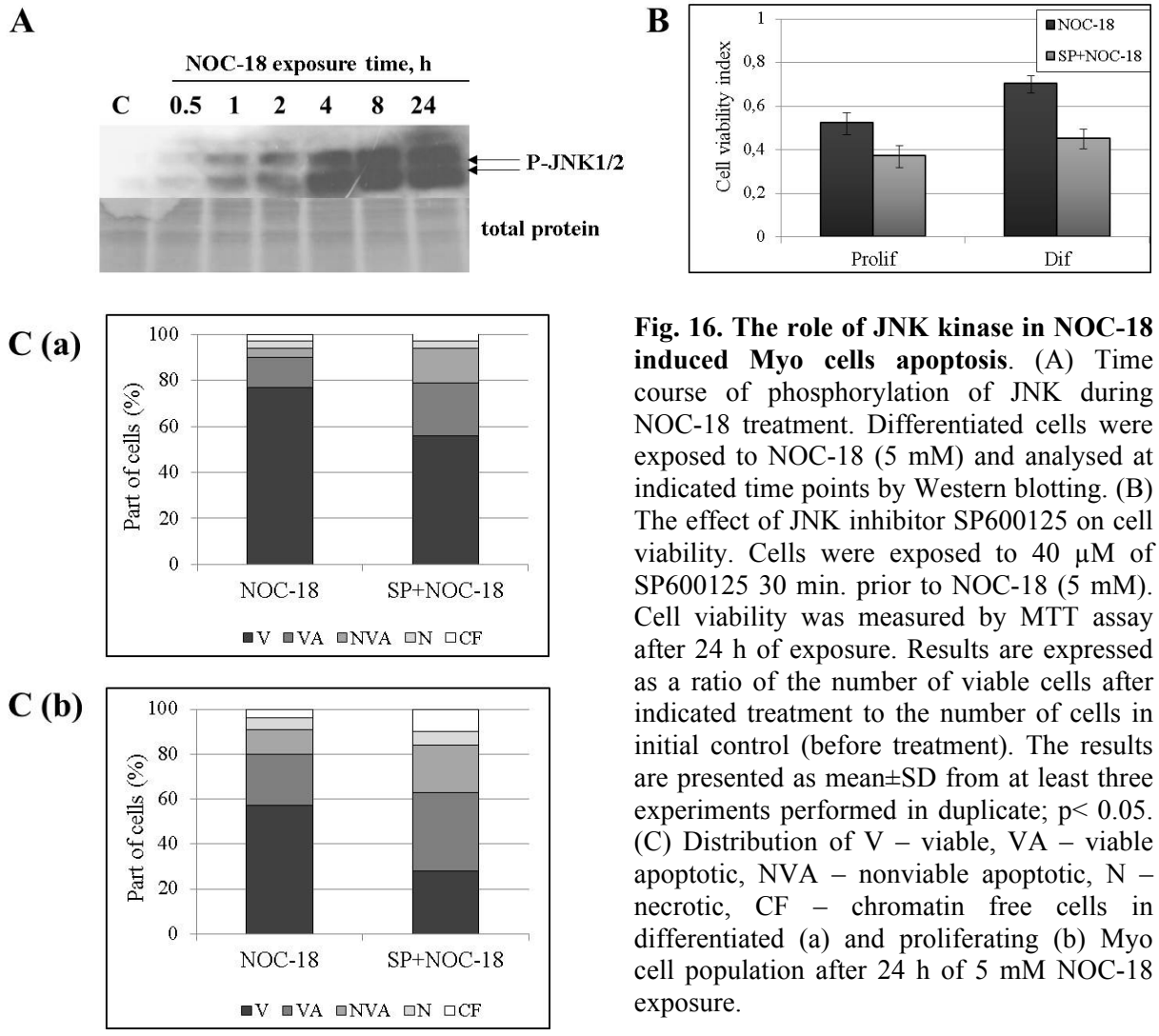


Fig. 16. The role of JNK kinase in NOC-18 induced Myo cells apoptosis. (A) Time course of phosphorylation of JNK during NOC-18 treatment. Differentiated cells were exposed to NOC-18 (5 mM) and analysed at indicated time points by Western blotting. (B) The effect of JNK inhibitor SP600125 on cell viability. Cells were exposed to 40 μM of SP600125 30 min. prior to NOC-18 (5 mM). Cell viability was measured by MTT assay after 24 h of exposure. Results are expressed as a ratio of the number of viable cells after indicated treatment to the number of cells in initial control (before treatment). The results are presented as mean±SD from at least three experiments performed in duplicate; $p< 0.05$. (C) Distribution of V – viable, VA – viable apoptotic, NVA – nonviable apoptotic, N – necrotic, CF – chromatin free cells in differentiated (a) and proliferating (b) Myo cell population after 24 h of 5 mM NOC-18 exposure.

Further we evaluated the role of JNK in regulating cisplatin induced apoptosis of Myo cells differentiated towards osteogenic, adipogenic and neurogenic cell lineage. Western blot analysis showed changes in phosphorylation of JNK and its downstream target c-Jun. As shown in Fig. 17, the treatment of differentiated cells with cisplatin resulted in gradual and sustained increase of JNK and its direct target c-Jun phosphorylation.

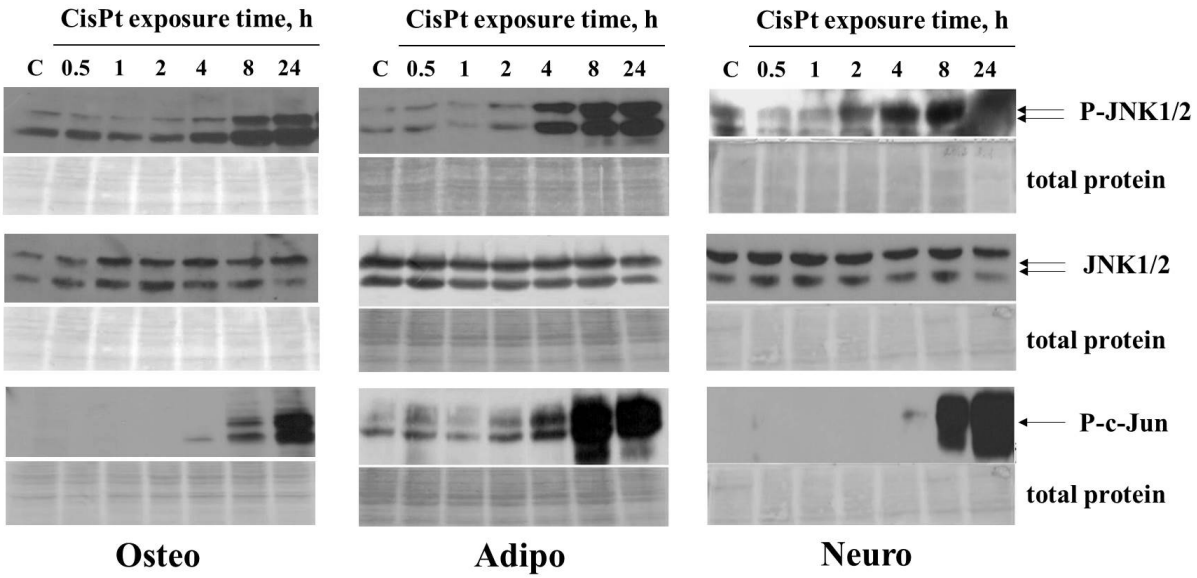


Fig. 17. Time course of JNK and c-Jun phosphorylation in response to cisplatin treatment. Osteogenically (Osteo), adipogenically (Adipo) and neurogenically (Neuro) differentiated Myo cells were exposed to 15 µg/ml cisplatin and analyzed at indicated time points. JNK and c-Jun phosphorylation was analyzed by immunoblotting with anti-phospho-JNK and anti-phospho-c-Jun antibodies. The total amount of JNK was assessed using antibodies that recognize these proteins regardless of their phosphorylation.

Our experiments applying JNK inhibitor SP600125, the effectiveness of which was confirmed by decreased c-Jun levels (Fig. 18B), show a protective role of JNK in Myo cells differentiated toward different lineages (Fig. 18A). Thus JNK acts as anti-apoptotic molecule in oste-, adipo- and neurogenically differentiated Myo cells, along with previously described myogenically differentiated cells.

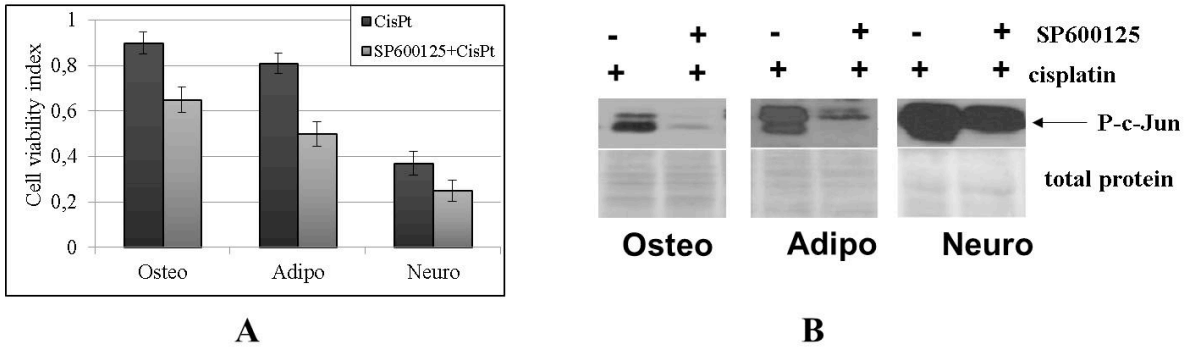


Fig. 18. The role of JNK in cisplatin induced Myo cell apoptosis. (A) The effect of JNK inhibitor SP600125 on differentiated cells viability. Osteogenically (Osteo), adipogenically (Adipo) and neurogenically (Neuro) differentiated cell were pretreated with 40 µM SP600125 30 min.prior to 15 µg/ml cisplatin. Cell viability was assessed by MTT method after 24 h of exposure. The results are expressed as a ratio of the number of viable cells after indicated treatment to the number of cells in initial control (before treatment). The data are presented as mean±SD from at least three experiments performed in duplicate; $p< 0.05$. (B) Effect of JNK inhibitor SP600125 on c-Jun phosphorylation. Cells were pretreated with 40 µM SP600125 30 min. prior to 15 µg/ml cisplatin. Phosphorylation of c-Jun was analysed by Western blotting with anti-phospho-c-Jun (Ser63) antibodies.

Extrinsic and intrinsic apoptosis initiation pathways are involved in cisplatin induced apoptosis of Myo cells The cellular process of apoptotic signal transduction is often split into two basic pathways: the extrinsic (receptor) and intrinsic (mitochondrial) ones. Both pathways are executed by a group of cysteinyl-proteases called caspases degrading multiple substrates within the cell (Baines et al., 2004). In order to evaluate cisplatin-induced apoptosis pathways in differentiated Myo cells we used specific caspase inhibitors. The results show that cisplatin induces apoptosis both through extrinsic and mitochondrial pathways (Fig. 19)

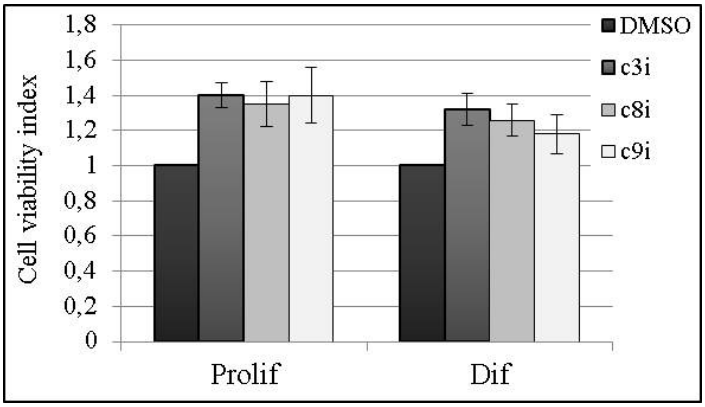


Fig. 19. Extrinsic and intrinsic apoptosis signal transduction pathways are involved in cisplatin-induced apoptosis of Myo cells. Proliferating (Prolif) and myogenically differentiated (Dif) cells were pretreated with caspase inhibitors c3i, c8i and c9i (all 20 μM). Cell viability was assesed by trypan blue assay after 24 h of exposure. The data are presented as mean±SD from at least three experiments performed in duplicate; $p< 0.05$. Cell viability index of DMSO and cisplatin-treated culture is taken to be a reference equal to 1.00.

Western blot data indicate an increase of Fas receptor expression in Myo cells exposed to cisplatin, but no change in FasL expression (Fig. 20A). The involvement of FasL/Fas pathway in cisplatin induced Myo cells apoptosis was also confirmed by treating the cells with an anti-Fas antibody that binds to the Fas antigen, a process that mimics the role of FasL in the induction of apoptosis. Simulation of the FasL action showed that FasL/Fas pathway is involved in cisplatin-induced Myo cell apoptosis (Fig. 20B).

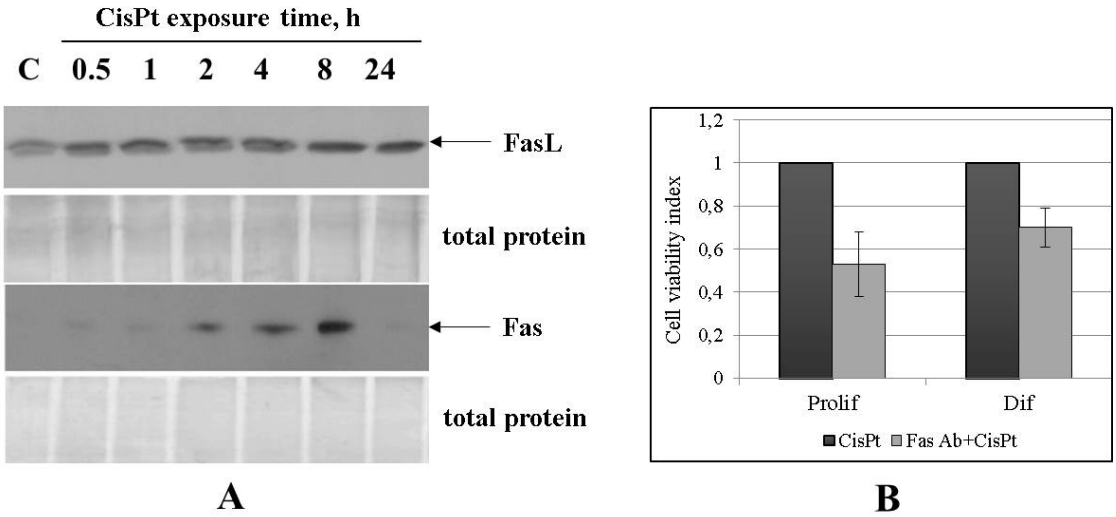


Fig. 20. The role of extrinsic FasL/Fas pathway in Myo cells apoptosis. A – Expression of FasL and Fas in cells exposed to 15μg/ml cisplatin at indicated time point was assessed by Western blotting. B – The effect of Fas antibodies on viability Myo cells. Cells were pretreated with antibodies against Fas for 30 min. before adding cisplatin. Cell viability was assessed by MTT assay after 24 h of exposure. The data are presented as mean±SD from at least three experiments performed in duplicate; $p< 0.05$. Cell viability index of solely cisplatin-treated culture is taken to be a reference equal to 1.00.

JNK is not involved in regulation of FasL/Fas pathway in differentiated Myo cells. The obtained data also show that pretreatment of proliferating cells with JNK inhibitor SP600125 leads to a decrease in Fas expression, whereas in myogenically differentiated cells there were not noticed any changes in Fas expression (Fig. 21). It may be concluded that JNK pathway may be not obligatory for Fas-mediated apoptosis in differentiated Myo cells.

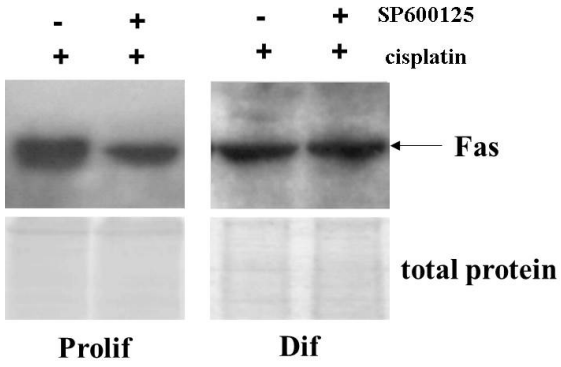


Fig. 21. Effect of JNK inhibitor SP600125 on Fas expression. Proliferating (Prolif) and myogenically differentiated (Dif) cells were pretreated with 40 μM SP600125 30 min.prior to 15 μg/ml cisplatin. Expression of Fas was analysed by Western blotting.

JNK is involved in regulation of intrinsic/mitochondrial pathway in Myo cells. There is increasing evidence of JNKs participation in regulating intrinsic/mitochondrial apoptosis pathway (Hatai et al., 2000; Soh et al., 2003). Potential targets of JNK may include members of the Bcl-2 family proteins, which are known to regulate cytochrome c release from mitochondria. Several studies indicate that these apoptotic regulatory proteins are phosphorylated by JNK with decrease in their antiapoptotic activity (Burke et al., 2007). However other researches showed an anti-apoptotic role of Bcl-2 phosphorylation by JNK (Deng et al., 2001; Ito et al., 1997; Davis, 2000), for example phosphorylation of antiapoptotic Bcl-2 on Thr74 and Ser87 residues was supposed to stabilize and protect protein from ubiquitin-proteolytic degradation. Activation of the Bcl-2 degradation process might therefore alter the balance between proapoptotic and antiapoptotic Bcl-2 members which is a critical determinant that plays a significant role in altering susceptibility to apoptosis (Breitschopf irk t., 2000; Oltvai et al., 1993).

In order to evaluate possible role of JNK in regulating mitochondrial apoptosis pathway we studied the expression of major regulators in the mitochondrial apoptosis pathway, i.e. antiapoptotic Bcl-2 and proapoptotic Bax protein in differentiated and proliferating cells pretreated with JNK inhibitor SP600125. The results of our study prove that JNK inhibition in differentiated cells exposed to cisplatin leads to a decrease in antiapoptotic Bcl-2 protein level. Simultaneously an increase of Bax expression was observed in differentiated cells pretreated with SP600125. In contrast, JNK inhibition in proliferating Myo cells had an opposite effect on change of Bax/Bcl-2 expression ratio. As shown in Figure 22, in proliferating cells SP600125 pushes Bax/Bcl-2 ratio down. It suggests that in Myo cells JNK exerts its role in apoptosis by regulating Bax/Bcl-2 ratio.

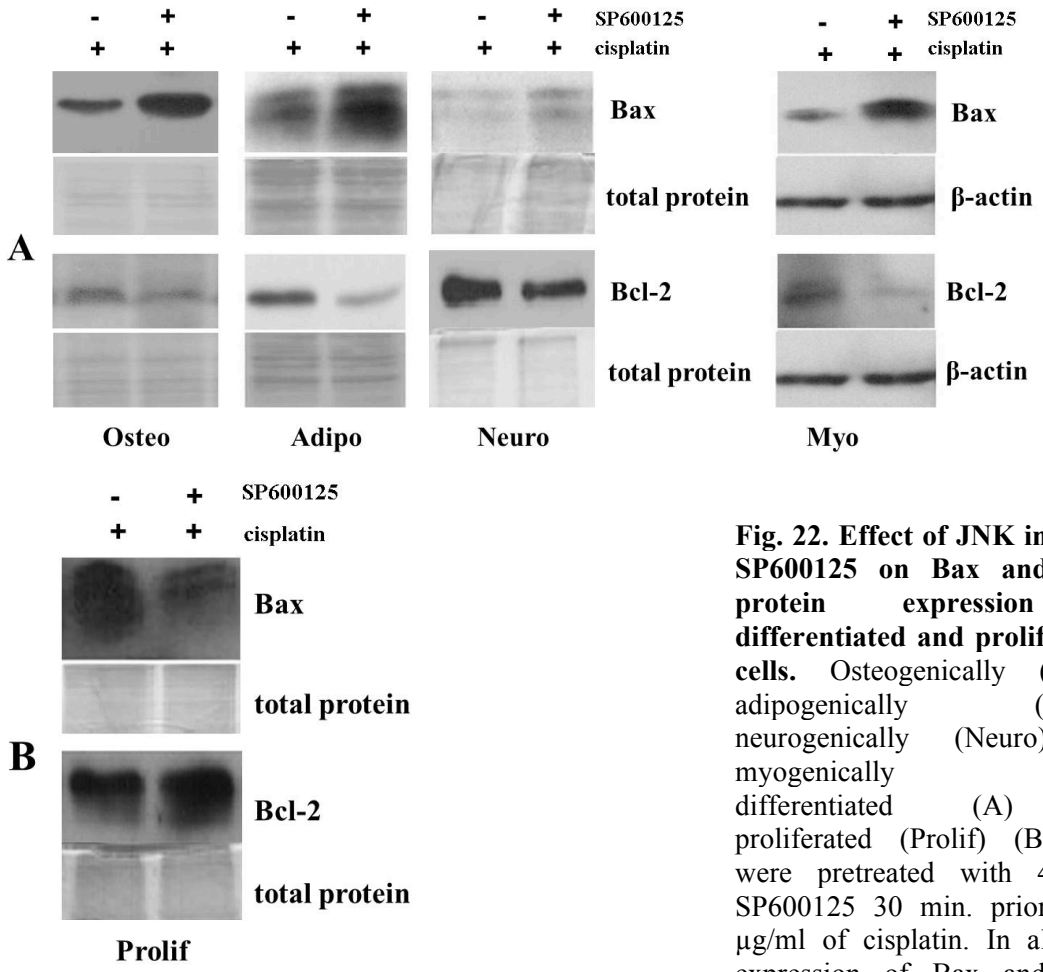


Fig. 22. Effect of JNK inhibitor SP600125 on Bax and Bcl-2 protein expression in differentiated and proliferating cells. Osteogenically (Osteo), adipogenically (Adipo), neurogenically (Neuro) and myogenically (Myo) differentiated (A) and proliferated (Prolif) (B) cells were pretreated with 40 μM SP600125 30 min. prior to 15 μg/ml of cisplatin. In all cases expression of Bax and Bcl-2 protein was analysed by Western blotting after 8 h of exposure to cisplatin.

JNK is involved in positive regulation of Akt kinase. Different mechanisms of the protective action of JNK have been reported, including the promotion of DNA repair mechanisms and involving cross-talk with other anti-apoptotic signalling pathways, such as PI3-kinase and Akt (Tran et al., 2007; Shao et al., 2006). There are data that JNK target protein c-Jun promotes cell survival by negatively regulating tumor-supressor PTEN and thereby activating the Akt (Hettinger et al., 2007).

In this study we proved a protective role of Akt in differentiated muscle-derived stem cells.

As our results show, pretreatment of differentiated cells with JNK inhibitor SP600125 results in a decrease of Akt phosphorylation. It is worth noting that in proliferating cells JNK inhibition did not affect Akt phosphorylation levels (Fig. 23), thus it should be excluded that JNK inhibitor directly inhibits Akt. It may be concluded that in differentiated cells JNK is involved in Akt kinase phosphorylation/activity regulation.

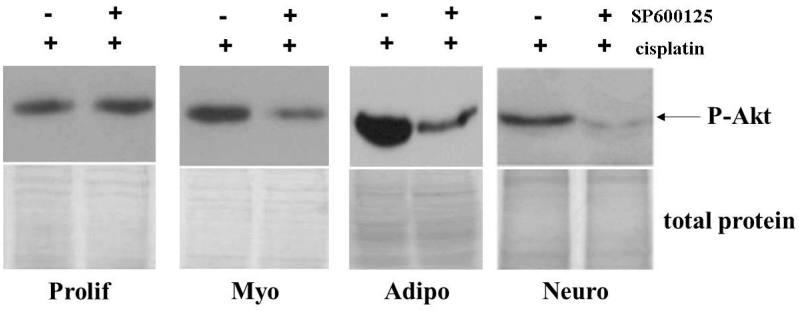


Fig. 23. Effect of JNK inhibitor SP600125 on Akt kinase phosphorylation. Proliferating (Prolif), myogenically (Myo), adipogenically and neurogenically (Neuro) differentiated cells were pre-treated with 40 μM SP600125 30 min. prior to 15 μg/ml cisplatin. Phosphorylation of Akt was analysed by Western blotting with anti-phospho-Akt (Thr308) antibodies after 8 h exposure to cisplatin

Consequently, adult muscle-derived Myo cell lines with unlimited proliferative potential *in vitro* are multipotent. These cells are not only able to differentiate into mesodermal lineage cells e.g. myogenic, adipogenic and osteogenic, but also possess the potential to break germ layer commitment and differentiate into neuron-like cells of ectodermal lineage. Myo-. adipo- and osteogenically differentiated cells become more resistant to genotoxic as well as oxidative stress. However differentiated neuron-like cells showed increased susceptibility to apoptotic treatment. Akt and MAPK signalling pathways are involved in survival of differentiated Myo cells exposed to cisplatin. JNK antiapoptotic action in differentiated cells may be explained by its ability to down-regulate mitochondrial apoptosis pathway and up-regulate activity of prosurvival kinase Akt. Therefore, applying appropriate drug therapy special attention should be paid to stem cell dynamics nature and cell response dependence on differentiation status.

Conclusions

- Adult rabbit muscle-derived cells with unlimited proliferative potential *in vitro*, are able to differentiate into myogenic, adipogenic, osteogenic and neurogenic cells.
- Myogenically, adipogenically and osteogenically differentiated cells are more resistance to apoptotic treatment than proliferating cells. Akt protein kinase protects differentiated cells from apoptosis.
- Cisplatin induce sustained activation of ERK and p38 in differentiated Myo cells. ERK and p38 protect differentiated Myo cell from apoptosis.
- The role of JNK in cisplatin and doxorubicin induced apoptosis changes from proapoptotic in proliferating cells to antiapoptotic in differentiated cells.
- JNK activation kinetics does not determine its role in apoptosis regulation of muscle-derived stem cells
- Antiapoptotic action of JNK kinase in differentiated cells may be associated with negative regulation of mitochondrial apoptosis pathway and positive regulation of Akt activity.

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Reziumė

Kamieninių ląstelių žūties ir išgyvenimo mechanizmų tyrimai yra svarbūs, siekiant išsaugoti organizme esančias kamienines ląsteles chemoterapijos metu bei taikant kamienines ląsteles regeneracinėje medicinoje pažeisto organo veiklos atstatymui. Šio darbo metu buvo charakterizuotos suaugusio triušio raumeninės kilmės ląstelės, pasižyminčios gebėjimu neribotai daugintis in vitro. Įvertinus ląstelių diferenciacinį potencialą, nustatytas jų daugiagališkumas, t.y. jos gebėjo diferencijuotis miogenine, osteogenine, adipogenine bei neurogenine kryptimis. Ši savybė išlieka ilgą laiką kultivuojant ląsteles in vitro. Tyrimų rezultatai patvirtina gautų miogeninių linijų ląstelių kamieninę prigimtį bei praplečia jų panaudojimo medicinoje galimybes. Griaučių raumuo yra patogus suaugusio organizmo kamieninių ląstelių šaltinis įvairiems raumeninio audinio bei neurologiniams susirgimams gydyti, kaulinio audinio regeneracijai.

Pirmą kartą nustatytos įvairiomis kryptimis diferencijuotų raumeninės kilmės kamieninių ląstelių žūties charakteristikos po genotoksinių ir oksidacinį stresą sukeliančių poveikių. Ląstelių citotoksiškumo tyrimai parodė didesnį miogenine, adipogenine bei osteogenine kryptimi diferencijuotų raumeninės kilmės kamieninių ląstelių atsparumą apoptozės sukelėjams, lyginant su proliferuojančiomis bei neurogenine kryptimi diferencijuotomis ląstelėmis.

Šiame darbe nustatytas Akt baltymų kinazės bei ERK, p38 ir JNK MAP kinazių aktyvinimo pobūdis po apoptozę indukuojančių poveikių bei įvertintas šių kinazių vaidmuo diferencijuotų miogeninių ląstelių apoptozėje. Nustatyta, kad Akt, ERK bei p38 apsaugo įvairiomis kryptimis diferencijuotas raumeninės kilmės kamienines ląsteles nuo apoptozės. Parodyta, kad skirtingas Akt fosforilinimo lygis diferencijuotose ląstelėse gali lemti nevienodą ląstelių jautrumą apoptozės induktoriams.

Tiriant JNK kinazės vaidmenį nustatyta, kad jos veikimas gali skirtis priklausomai nuo apoptozės induktoriaus bei ląstelių diferenciacijos stadijos. Parodytas JNK vaidmens pasikeitimas iš proapoptozinio, proliferuojančių ląstelių atveju, į antiapoptozinį, ląstelių diferenciacijos bei kontaktinio augimo metu po priešvėžinių vaistų cisplatinos ir doksorubicino poveikio. Tyrimų duomenys rodo, kad JNK vaidmuo ląstelių apoptozėje nėra nulemtas jos aktyvinimo kinetikos bei trukmės. Tiek proliferuojančiose, tiek ir diferencijuotose ląstelėse nustatytas palaipsnis ir ilgalaikis JNK fosforilinimas.

Aiškinantis JNK veikimo diferencijuotose Myo ląstelėse mechanizmus, nustatyti galimi jos antiapoptozinio veikimo taikiniai - parodyta, kad JNK apsauginis vaidmuo gali pasireikšti per mitochondrinio apoptozės kelio slopinimą bei išgyvenimo kinazės Akt aktyvinimą.

Tyrimo rezultatai leidžia padaryti išvadą, kad siekiant apsaugoti kamienines ląsteles nuo toksinio vaistų poveikio įvarių susirgimų gydymo metu, svarbu atsižvelgti į .kamieninių ląstelių diferenciacijos stadiją bei jos kryptį.

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